Purification of Herpes Simplex Virus

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SUMMARY

Herpes simplex virus was highly purified from host cell material with little loss of infectivity by the combination of isopycnic centrifuging in a three layered silica gradient and rate zonal centrifuging in dextran T 70 gradient. By layering three solutions of colloidal silica (24, 18 and 12%, w/v) on top of each other in the centrifuge tube prior to ultracentrifuging, a linear type of gradient was obtained. Virus particles and nucleocapsids seemed to be distinctly separated in this three-layered silica gradient. The overall reduction of host proteins and sphingolipids was 1250 to 2000 times and 350 to 600 times, respectively. The reduction of virus specified non-particulate proteins was approx. 500 times. The recovery of infectivity was 55 to 70%.

Purification of large enveloped virus particles is often associated with substantial loss of infectivity. Envelopes of herpes viruses are sensitive to drying and to mechanical treatment such as centrifugal stress and to the influence of the surrounding medium. Procedures of purification including sucrose gradient sedimentations are in general deleterious for herpes viruses and great losses of infectivity are encountered (McCombs, 1969; Ben-Porat, Shimono & Kaplan, 1970; Robinson & Watson, 1971). Rate zonal centrifuging using dextran gradients (Spear & Roizman, 1972) and isopycnic centrifuging in colloidal silica (Pertoft, 1970a, b; Klingeborn & Pertoft, 1972) were not deleterious to the same extent to envelopes and infectivity.

The present study took advantage of both these observations. The procedure developed for the purification of HSV involved a technique of centrifuging in colloidal silica in combination with centrifuging in a dextran T 70 gradient. The dextran gradient centrifuging served both as a further step of purification and as a method to free the virus preparations from silica. To label cellular proteins uninfected GMK cells were cultivated in MEM without leucine supplemented with calf serum and [3H]-leucine. Cellular DNA was labelled by growing GMK cells in MEM supplemented with calf serum and [3H]-thymidine. Cellular lipids were labelled by growing GMK cells in MEM supplemented with [14C]-labelled tetracosanoic acid methyl ester (J. Blomberg & N. Dinh-Nguyen, unpublished observations) dispersed in foetal calf serum. Cells were harvested on the 4th to 5th day. Virus proteins were labelled by incubating infected cells in MEM without leucine then adding [3H]-leucine 5 to 7 h post-infection. To label virus DNA, finally, infected cells were incubated in MEM adding [3H]-thymidine 5 to 7 h post-infection. Cells were harvested 18 to 24 h after infection. The cytoplasmic fraction of infected cells, separated from nuclei by the method described by Spear & Roizman (1972), served as source of virus particles. Thus, infected cells were harvested 18 to 24 h post-infection and centrifuged at 1500 rev/min. The sediment was resuspended in 1 mm-phosphate buffer, pH 7.4, and disrupted in a tight fitting Dounce homogenizer. Immediately afterwards, 1/8 parts of 60% (w/v) sucrose was added. Nuclei were removed from the cytoplasm by another low speed centrifuging.

Isopycnic centrifuging was performed in colloidal silica (Ludox HS, 40% SiO_2 (w/v),
Fig. 1. Ludox HS solutions centrifuged in a Spinco SW 25.1 rotor at 52000 g for 60 min. ▲—▲, 20% (w/v) Ludox; •—•, 13% (w/v) Ludox layered on top of 27% (w/v) Ludox; ○—○, 10.5 ml 13% (w/v) Ludox, 6 ml 20% (w/v) Ludox and 10.5 ml 27% (w/v) Ludox layered on top of each other.

Fig. 2. An artificial mixture of the cytoplasmic fraction from HSV infected cells and [3H]-leucine-labelled uninfected cells was mixed with the silica gradient solution in the proportions 1:2, 1:1 and 2:1. These solutions were layered on top of each other in centrifuge tubes and were centrifuged in a Spinco SW 25.1 rotor at 52000 g for 70 min (a). The virus fractions from the experiment described above were diluted with 2 parts of 1 mm-phosphate buffer and layered on top of dextran T70 density gradients, 11 to 38% (w/v). Centrifuging was performed in a Spinco SW 25.1 rotor at 40000 g for 2 h (b). Fractions were assayed for radioactivity (●—●), density (▲—▲) and virus infectivity (○—○).

E.I. du Pont de Nemours, Wilmington, Md., U.S.A.). The gradient solution consisted of Ludox HS (36% SiO₂, w/v), pH 8.0, 5% (w/v) polyethylene glycol (PEG 4000) and 0.1% (w/v) bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, England).

The cytoplasmic extract was mixed with the gradient solution in the proportions 1:2, 1:1 and 2:1, giving mixtures containing 24, 18 and 12% (w/v) Ludox, respectively. These mix-
Table 1.

<table>
<thead>
<tr>
<th>Centrifuging conditions</th>
<th>Reduction of host protein</th>
<th>Reduction of host sphingolipids</th>
<th>Reduction of virus-specified proteins</th>
<th>Recovery of infectivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-speed centrifuging of Dounce homogenized infected cells</td>
<td>× 5</td>
<td>× 5</td>
<td>× 5</td>
<td>100</td>
</tr>
<tr>
<td>Centrifuging in silica</td>
<td>× 21–36</td>
<td>× 6–10</td>
<td>× 6</td>
<td>70–80</td>
</tr>
<tr>
<td>Centrifuging in dextran</td>
<td>× 10–12</td>
<td>× 24</td>
<td>× 15–20</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>Total</td>
<td>× 1250–2000</td>
<td>× 350–600*</td>
<td>× 450–600</td>
<td>55–70</td>
</tr>
</tbody>
</table>

* Corrected for 50% incorporation of the labelled fatty acid.

tures were then layered sequentially on top of each other in 3 × 1 in. centrifuge tubes (10.5 ml, 6 ml and 10.5 ml, respectively) and centrifuged in a Spinco SW 25.1 rotor at 52000 g for 70 min. Four bands were observed and all virus infectivity was found in the second band from the top. Rate zonal centrifuging was performed through dextran T 70 (Pharmacia, Uppsala, Sweden) density gradients, 11 to 38% (w/v). The band which contained virus after isopycnic centrifuging in silica was diluted with 2 parts of the 1 mM-phosphate buffer, layered on top of the dextran gradients and centrifuged at 41000 g for 120 min in a Spinco SW 25.1 rotor. Virus infectivity was recovered somewhat below the middle of the gradient, and was separated from most of the silica which sedimented to the bottom of the tube.

Density of the fractions from the Ludox and dextran gradients was tested by the method described by Miller & Gasek (1960).

Because of its polydispersity colloidal silica has the property of generating a density gradient during centrifuging (Pertoft, 1966; Pertoft et al. 1967). The gradient formed changed during centrifuging and soon became steep at the bottom of the tube.

In order to construct a suitable silica gradient, Ludox solutions of different concentrations were layered on top of each other and were centrifuged in a Spinco SW 25.1 rotor at 52000 g for 60 min. Fig. 1 shows three different types of Ludox gradients. A linear type of gradient was formed when three Ludox solutions of 13, 20 and 27% (w/v), respectively, were layered on top of each other. This type of gradient was used in the procedure of purification used.

In a series of experiments, mixtures of HSV and [3H]-leucine labelled and homogenized GMK cells were centrifuged in gradients of silica and the band containing virus was subsequently submitted to rate zonal centrifuging in dextran T 70 density gradients. Samples of fractions from sedimentations containing the virus and samples from other preparatory steps were assayed for radioactivity and virus infectivity. The results are shown in Table 1 and in Fig. 2. Host protein radioactivity was reduced 21 to 36 times by the centrifugings in silica gradient. The subsequent dextran gradient centrifuging reduced the proportion 10 to 12 times further. The recovery of infectivity after the centrifugings was 70 to 80% and more than 80% respectively. Thus the total reduction in protein of cellular origin was 1250 to 2000 times and the total recovery of infectivity 55 to 70% (Table 1). When subjected to 100000 g for 2 h, 80% of the radioactivity in the fraction of the dextran gradient containing virus sedimented.

About 50% of the lipid-extractable radioactivity was found in the sphingolipids of the GMK cells incubated with [3H]-tetracosanoic acid methyl ester.

The cytoplasmic fraction of homogenized GMK cells labelled with [3H]-lipids was mixed with HSV, and centrifuged in the silica-PEG and dextran gradients as described above.
Centrifuging in silica gradient removed cellular lipids 6 to 10 times and the dextran gradient centrifuging 24 times. The total reduction of radioactivity was 700 to 1200 times (Table I).

In the cytoplasmic extract of HSV infected cells, labelled with $[^3]H$-leucine 5 h after inoculation of virus, the radioactivity corresponds not only to virus particles and nucleocapsids, but also to membrane glycoproteins, non-structural proteins and structural non-assembled proteins (Spear & Roizman, 1972). Two peaks were observed after centrifuging in the silica gradient; one at a density of 1.09 to 1.11 g/ml and the other at a density of 1.13 to 1.15 g/ml. The authors assumed that the first peak corresponded to the enveloped virus particles and the latter to nucleocapsids. The overall recovery of labelled, presumably virus-specified proteins, in the virus fractions was 3.4% after the silica gradient centrifuging and approx. 0.2% after the dextran gradient centrifuging (Table I).

The distribution of DNA from infected and uninfected cells in the silica gradient was studied. The crude nuclear fraction, separated from most of the cytoplasm as described, was diluted with 1 mM-phosphate buffer and homogenized in a tight-fitting Dounce homogenizer with 25 strokes. By this treatment the nuclear membranes were disrupted and the nucleocapsids released. The homogenate obtained contained labelled nucleocapsids and virus particles. The results of centrifuging in the silica gradient of homogenized nuclei are shown in Fig. 3(a). The profile of radioactivity indicated three peaks, corresponding to densities ranging from 1.13 to 1.15, 1.09 to 1.11 and 1.06 to 1.08 g/ml, respectively.

In another experiment the sedimented nuclear fraction was resuspended in Hanks's BSS and centrifuged at 1500 rev/min for 15 min to remove remaining cytoplasm. The nuclei homogenized as above were centrifuged a second time at 2000 rev/min for 20 min to remove cellular debris. This was done to increase the proportion of nucleocapsids relative to virus particles. The results of the sedimentations are presented in Fig. 3(b). The peak observed at
the density of 1.13 to 1.15 g/ml was proportionately higher than the peak at 1.09 to 1.11 g/ml. The third peak previously detectable at a density of 1.06 to 1.08 g/ml was not observed. It seemed thus, as if the third peak corresponded to virus material attached to nuclear membranes or to receptors of the plasma membranes, sedimentable by low-speed centrifuging.

These findings also indicated that nucleocapsids and enveloped viruses banded separately and that the first peak corresponded to nucleocapsids and the second to enveloped viruses. Control materials of uninfected labelled GMK cells processed as described in the first of the two previous experiments demonstrated two peaks of radioactivity, one at 1.09 to 1.11 g/ml and the other at density of 1.13 to 1.15 g/ml. Low-speed centrifuging of the homogenized nuclear fraction removed 94% of the radioactivity from the supernatant fraction. After centrifuging the supernatant fluid in a silica gradient the radioactivity was uniformly distributed in the gradient.

Thus the silica gradient distinctly separated virus particles and nucleocapsids. Preliminary electron microscopic observations also supported this conclusion.

Proteins synthesized from 4 to 5 h post-infection are specified by the virus (Sydiskis & Roizman, 1967; Spear, Keller & Roizman, 1970), and labelled thymidine is incorporated into virus DNA mainly when cells are incubated with the nucleoside 6 to 17 h post-infection (Kaplan & Ben-Porat, 1963; Roizman & Roane, 1964). The distribution of proteins and DNA, assumed to be virus-specific in the silica gradient, contrasted with the distribution of similarly treated cellular proteins and DNA in a manner consistent with these findings. Cellular proteins of non-infected cells labelled with [3H]-leucine were distributed throughout the gradient, whereas radioactivity of infected cells labelled 5 h after infection appeared in two peaks. When uninfected homogenized cells labelled with [3H]-thymidine were utilized, two peaks appeared: one at 1.09 to 1.11 and another at 1.13 to 1.15 g/ml. Both disappeared after low-speed centrifuging. Cells incubated with the nucleoside 6 to 17 h after HSV infection yielded two peaks appearing at the same density ranges as above, and, in addition, a major third peak at 1.06 to 1.08 g/ml (Fig. 3a). After low-speed centrifuging this third peak disappeared, but the two other peaks remained (Fig. 3b). None of these three peaks should consequently correspond to cellular DNA.

The difference in degree of purification of the virus with regard to proteins and lipids demonstrates the necessity of testing the purity of virus preparations with respect to more than one class of substances. Thus results based on analyses of one class of substances may be erroneous with regard to the degree of purity of another.

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